STUDIES ON OIL PRODUCING EXTREMOPHILES FROM LONAR LAKE

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INTRODUCTION:

Microbial oil is commonly defined as lipids that are accumulated by oleaginous microorganisms. In general, oleaginous microorganisms are able to accumulate intracellular oil at more than 20% of their cell dry weight. Many microorganisms that belong to the genera of yeast, fungi, bacteria and algae have been reported to accumulate significant amounts of lipids during fermentation on various renewable resources. Single cell oils (SCOs) are intracellular storage lipids comprising of triacylglycerol (TAGs). SCOs are produced by oleaginous microorganisms which can accumulate between 20% and up to 80% lipid per dry biomass in the stationary growth phase under nutrient limitations, e.g., nitrogen or phosphorus, with simultaneous excess of carbon source. Depending on the oleaginous microorganism including bacterial, yeast, microalgae or fungal species, fatty acid profile of SCOs can vary making them highly suitable for diverse industrial applications.

Although the prospects of obtaining useful, and possibly even cheap, oils from microorganisms have been considered for nearly a century, it is only in the past two or three decades that they have begun to be produced commercially. All the oils in current production are high in their contents of polyunsaturated fatty acids (PUFA) and are destined mainly for human consumption as nutraceuticals though some are used for animal feeding, including feed for farmed fish. More recently, however, microbial oils have begun to be considered as possible sources of biofuels. Both heterotrophic organisms (yeasts and fungi) as well as photosynthetic algae are being actively pursued as possible production organisms.

Microbial oil accumulation in yeasts fulfils energy storage purposes and it consists of triglycerides (TAGs) which are produced in the endoplasmic reticulum and lipid bodies in the cytosol. Oleaginous yeasts can utilize a wide range of carbon sources for *de novo* lipid biosynthesis. Microbial oil production is triggered under nutrient limiting conditions leading to accumulation of citric acid in the mitochondrion that is subsequently secreted into the cytoplasm where it is converted to acetyl-CoA (the precursor for fatty acid synthesis) and oxaloacetate by ATP citrate lyase [1]. Various

substrates can be valorised by oleaginous yeasts for microbial oil production such as pure sugars, sugar-rich wastes (molasses and cheese whey), lignocellulosic materials, biodiesel derived glycerol, starch and food-processing wastes.

Microbial oil production by oleaginous yeasts can be used for biodiesel production and as a raw material for oleochemical production such as detergents, soaps, plastics, surfactants, lubricants, paints, and additives for the food and cosmetic industry among others [1], [5]. Biodiesel is a renewable, biodegradable and non-toxic energy resource that can be produced by transesterification of triacylglycerols (from edible and non-edible oil-rich feedstocks) with methanol to produce fatty acid methyl esters. However, due to the continuously increasing demand for first generation biodiesel and the fact that biofuel production should depend on non-edible feedstocks, researchers focus on the production of microbial oil as an alternative feedstock for biodiesel production. Microbial oil produced by oleaginous microorganisms has unique fatty acid composition which is affected by the type of carbon source, nature of microorganism and the cultivation conditions. In most cases, the fatty acid profile is similar to plant and animal oils, containing mainly C16 and C18 fatty acids. To evaluate the suitability of biodiesel production from microbial oil, several properties should be analyzed.

In the current global scenario, the world is under a serious dilemma due to the increasing human population, industrialization, and urbanization. The ever-increasing need for fuels and increasing nutritional problems have made a serious concern on the demand for nutrients and renewable and eco-friendly fuel sources. Currently, the use of fossil fuels is creating ecological and economic problems. Microalgae have been considered as a promising candidate for high-value metabolites and alternative renewable energy sources. Microalgae offer several advantages such as rapid growth rate, efficient land utilization, carbon dioxide sequestration, ability to cultivate in wastewater, and most importantly, they do not participate in the food crop versus energy crop dilemma or debate. An efficient microalgal biorefinery system for the production of lipids and subsequent by product for nutraceutical applications could well satisfy the need. But the current microalgal cultivation systems for the production of lipids and nutraceuticals do not offer techno-economic feasibility together with energy and environmental sustainability.

Microalgae are microscopic photosynthetic organisms and primitive eukaryotic plants on the planet earth. They are unicellular to multi-cellular organisms with different sizes ranging from one to hundred microns. Microalgae have different habitats like freshwater and ocean systems and they play the role of primary producers in the food chain (Udayan et al. 2021). These biological cell factories are the natural nutritional base and primary source for the aquatic food chain. Microalgae are primitive plants belonging to Thallophytes without stems, leaves, and roots and have *chlorophyll a* as primary photosynthetic pigment. Other examples of Thallophytes are fungi, lichens, and some classes of bryophytes, bacteria, and slime molds. Among these Yeast and microalgae are widely studied for biofuel applications. Photosynthetic machinery of land plants is evolved from microalgae and are considered as the primary producers of the aquatic ecosystem. From ancient times, microalgal biomass has been utilized to produce fuels, food, medicine, etc.

In the present global scenario, the major fuel sources used are petrol, diesel, coal, and natural gas (Enamala et al. 2018). At the current rate of consumption and overexploitation, it is estimated that the existing fossil fuels will be depleted in the coming 50 years (Martins et al. 2019). The level of greenhouse gases such as CO_2, CH_4, and N_2O have increased tremendously in the atmosphere to 409.8 ppm, 1875 ppb, and 331.1 ppb as of 2017 compared to the 1800s, which was 289.9 ppm, 750.8 ppb, and 273 ppb, respectively (Köhler et al. 2017). This alarmingly high increase in CO_2 and other greenhouse gas emissions could be reduced by using renewable liquid biofuels.

Liquid biofuels can be used without any major changes compared to the other renewable energy sources such as solar, tidal, and wind energy generation and release of toxic gases into the environment, which contributes to a major reason for global warming which is the major drawback of fossil fuels. The importance of biofuels has increased recently due to the above-mentioned reasons. Biofuels are produced from different biomass sources from forestry, agricultural and aquatic sources.

Microalgae have a high rate of lipids production per hectare yield which is 7–31 times higher than other oil crops. Microalgal biofuels are non-toxic, highly biodegradable with no sulfur content. The utilization of CO_2 by algae during the growth

makes it a feasible CO_2 mitigation agent from power stations and industrial plants, which could lead to environmental pollution (Sreekumar et al. 2018; Joun et al. 2021; Sirohi et al. 2021b). Microalgae represent primitive land plants and depend on photosynthesis to produce chemical energy from solar energy. The chemical energy synthesized by microalgae is stored in the oils, proteins, and carbohydrates. While considering biofuels, the lipid yield from a particular species highly affects its energy production efficiency (Sreekumar et al. 2016). Microalgae have a higher growth rate, high photosynthetic efficiency, adaptability to harsh environmental stress conditions, and can grow in wastewater with high nutrient uptake ability.

The present study will focus on the production of lipids and nutraceuticals from microalgae and yeast, covering the current strategies used for lipid production and the major high-value metabolites from microalgae and their nutraceutical importance. This study will also focus on the future strategies for enhanced microalgal lipid production and subsequent utilization of microalgal biomass.

Review of literature (Previous work done)

The use of fossil fuels has been strongly related to critical problems currently affecting society, such as: global warming, global greenhouse effects and pollution. These problems have affected the homeostasis of living organisms worldwide at an alarming rate. Due to this, it is imperative to look for alternatives to the use of fossil fuels and one of the relevant substitutes are biofuels. There are different types of biofuels (categories and generations) that have been previously explored, but recently, the use of microalgae has been strongly considered for the production of biofuels since they present a series of advantages over other biofuel production sources: (a) they don't need arable land to grow and therefore do not compete with food crops (like biofuels produced from corn, sugar cane and other plants) and; (b) they exhibit rapid biomass production containing high oil contents, at least 15 to 20 times higher than land based oleaginous crops. Hence, these unicellular photosynthetic microorganisms have received great attention from research to use them in the large-scale production of biofuels. However, one disadvantage of using microalgae is the high economic cost due to the low yields of lipid content in the microalgae biomass. Thus, development of different methods to enhance microalgae biomass, as well as lipid content in the microalgae cells, would lead to the development of a sustainable low-cost process to produce biofuels. Within the last 10 years, many studies have reported different methods and strategies to induce lipid production to obtain higher lipid accumulation in the biomass of microalgae cells; however, there is not a comprehensive review in the literature that highlights, compares and discusses these strategies. Here, we review these strategies which include modulating light intensity in cultures, controlling and varying CO_2 levels and temperature, inducing nutrient starvation in the culture, the implementation of stress by incorporating heavy metal or inducing a high salinity condition, and the use of metabolic and genetic engineering techniques coupled with nanotechnology. (*Microbe Cell Fact* **18**, 178)(2019)

Increased oil prices, depletion of fossil fuels and high energy demand have made the researchers to look for an alternative source. Algal fuel might be an alternative to fossil fuel because these feedstocks are much more reliable and using them as biofuel production can decrease the dependence on fossil fuels (Bansal and Sharma, 2005; Kulkarni and Dalai, 2006). Many algae biofuel studies have shown that the quantity and quality of lipids within the algal cell biomass can vary as a result of

changes in growth conditions (temperature, pH and light intensity) or nutrient media characteristics (concentration of nitrogen, iron and phosphates) (Illman et al., 2000; Liu et al., 2008).

Over the past few decades, concerns about food safety and agricultural sustainability have increased and microalgae have gained interest as natural substances rich in a variety of bioactive compounds, including lipids, proteins, amino acids, and polysaccharides. Among them, lipids are a good replacement for fish oil because of its high PUFAs content. Microalgae lipids extraction has been studied more specifically considering the effects on the cell walls. This paper summarizes the techniques and assists techniques of microalgae lipids' extraction in order to provide more comprehensive information about the topic, and to identify opportunities and challenges to overcome. Classical extraction techniques (Soxhlet, Folch, Bligh-Dyer, etc.) have been widely used and fully explored. Although most of them are simple to operate, they also cause damage to the environment due to the extensive use of toxic reagents and the extraction time is long. Therefore, it is necessary to explore efficient and environmentally friendlier methods. A series of J. Zhou et al. As an important source of PUFAs in the ocean, microalgae have attracted more and more attention of researchers. Therefore, is of great interest to explore appropriate techniques to improve the extraction rate and reduce the cost on the premise of ensuring the bioactive abilities of lipids, so that microalgae can be better applied to industries. In the future, the application of microalgae on a larger scale is worth expecting. In addition to combining existing technologies and develop new technologies, it is also necessary to further explore the use of bio-based non-toxic extractants in the extraction process. This makes it easier for the target components to be extracted to be applied, as well as recycling of the remaining material after extraction. Moreover, algae biomass fractionation, in the scope of algae biorefinery approach for high-value compounds extraction, is expected to boost in the next years. (*Article in Biotechnology (Faisalabad) · June 2011)*

Screening and evaluation of potential green algal strains viz biomass productivity, lipid cell content, fatty acids and fuel properties are some of the key parameters that determine the economic feasibility of algal oils for biodiesel production. In this work, nine Chlorophyceae strains isolated from freshwater and soda lakes from Central Europe were compared according to their biomass and lipid

productivities. The total lipids ratios in dry biomass varied from as low as 8.9% to ratios as high as 42.1%. The highest lipid yields were observed from the C. vulgaris LC8 strain with a favourable C16-C18 fatty acid profile of 77.38%, as well as desirable biodiesel properties like a high cetane number (57.3), low viscosity (4.67 mm2 /s), lower iodine number (75.2 g I2/100 g), relative cloud point (8.8 °C) and negative cold filter plugging point (−6.5 °C). C. vulgaris LC8 presented one of the best combinations of desirable traits, which makes it a good candidate for further assessment for biofuel production. Results suggest that the adequate fatty acid composition of microalgal oil and the lipid productivity of strains must be priority criteria for strain selection, to make the algae-based biodiesel industry viable. (Ren, X., Wei, C., Yan, Q. *et al. Sci Rep* **11**, 20221) (2021)

From the present work it can be suggested that Spirogyra sp. and Oedogonium sp. and Chlorella sp. are common species of Lahore. Each of the algal species requires a different set of growth conditions if cultured in the laboratory. The comparison of oil contents indicate that Chlorella sp. has a higher oil content as compared to other algal species tested during the present *work. (Journal of Animal and Plant Sciences · April 2015)*

The expanding body of microalgal ALE experimental work targeting improved growth rate, increased stress tolerance, novel substrate utilization, and greater product yield, can inform the design of future experiments and further advances in this field. Successful microalgal ALE experiments thus far have validated the methods originally developed in microbial systems, and, through the use of automated culturing techniques and next-generation sequencing methods, future studies can achieve additional and more complex adaptations to ultimately assist in the development of microalgal bioproducts and biofuels. (*Alaina J. LJapanese, Anagha Krishnan, Matthew C. Posewitz, j. algal.2020.102122).*

OBJECTIVES OF RESEARCH:

Following Objectives are proposed for present research work-

1.Isolation, Identification and Characterisation of oil producing Algae.

2.Screening of their Oil Producing Potentials.

3. Optimisation of oil production for better yield

4. Strain improvement by Natural selection for better yield

5. Characterisation of lipids/oils by modern analytical methods

Materials and methods

Isolation & characterisation of oil producing Algae

Sample collection: During the present study, samples will be collected from lonar lake from buldhana district. The samples can be collected throughout the year. It will be collected in glass jars, Plastic bags and bottles and then transferred to various nutrient media standardised in lab.

Culture Media Preparation: Six salts (10g of sodium nitrate, 1g of calcium chloride, 3g of magnesium sulphate, 3g of potassium phosphate monobasic, 7g of potassium phosphate dibasic, and 1g sodium chloride) each will be dissolved in 400 mL of distilled water or deionized water and stirred. Then 10 ml from each of the salt solution will be measured in to 900 ml of distilled water. Thereafter, 15g of agar will be added into the flask containing salt solution of the 900 ml distilled water and mixed. It will be then covered and autoclaved. This was later stored at a refrigerated temperature after use, for further use within a period of two weeks (Bristol medium, 2011).

Isolation of Microalgae from the Pond Water: Microalgae isolation will be done using serial dilution to obtain pure culture from water sample by employing Bristol's Medium using pour plate. 1 ml of the water sample from the pond will be introduced into sterilized labelled test tubes containing 9ml distilled water. One (1ml) aliquot of the diluents will be introduced into labelled sterile Petri-dishes aseptically and spread all over using the spreader. Fifteen (15) ml of sterilized Bristol's Medium will be poured aseptically into the different already prepared labeled Petri-dishes and allowed to solidify in the laminar flow chamber. The Petri-dish will be sealed up using paraffin paper to avoid contamination. It was left beside the window for source of light energy for two (2) weeks (Abirhire and Kadiri, 2013).

Screening of oil producing potentials

Isolation and Identification of Pure Algal Strain: Isolation of pure algal strain from cultured plates will be carried out using streak plate method. Petri dishes containing sterilized growth medium solidified with 1-1.5% Bristol's medium will be prepared. Colonies free of other organisms will be selected for further isolation from a mixed

cultured plate. Using aseptic technique, the sterile loop will be used to make parallel streaks of the colonies on the solid medium. The plate will be covered and sealed with parafilm. It was inverted, then incubated under low light (window light reflection) at constant temperature for about 2 weeks. When growth is observed on the plate, a sample will be removed using a sterilized wire loop, capillary tube and placed in a drop of water on a slide and covered with a glass-slide which will be checked microscopically to ensure pure algal isolates (Shah et al., 2003).

Identification of Microalgae: Microalgae will be identified in the laboratory with the aid of a microscope. Morphological features of the algae were compared with the algal compendium and identification keys found in the freshwater algae. Microscopic observation of the various algal isolates confirmed for their purity and allowed for preliminary identification of isolates for their classification into various classes based on the morphological characters.

Cultivation: Blue-green (BG) medium and Bold basal (BB) medium (Bold 1949) will be tested for the algal growth and support. These growth medium are mostly adapted to freshwater algae. Growth experiments will be performed in conical flasks of 250 ml. Each flask will be filled with 100 ml of media and initially 2 g of algal sample will be added to each flask. Every experiment will be performed in replicates and both the media were compared for each culture conditions.

Optimization of production for better yield

Optimization of different culture conditions: The effect of different parameters on the growth rate of algae will be assessed by following completely randomized experimental design. In these experiments only one factor will be variable while all other conditions will be kept constant. The culture conditions which were controlled for the algal growth were nutrients, light, temperature, pH and constant mixing or aeration.

Culture medium/Nutrients: For monitoring the best growth of algae both the media (BB and BG) will be used for culturing algal cells and the best growth rate will be estimated after a required period of time has been passed. The first calculation will be made on 7th day and the rate will be estimated on the basis of the rate of increase in fresh weight of biomass. The cell mass will be separated through filtration and then weighted after blotting the excess water.

Temperature: The temperatures will be maintained at 16, 20, 24, 28 and 32^{o}C. Temperature variations were monitored regularly using thermometer. Changes will be recorded and the algal growth rates will be found by measuring their fresh weights.

pH: The culture media will be adjusted with various pH ranges i.e., 6.5, 7, 7.5, 8, 8.5, and 9. Algae samples will be grown within these pH ranges and the effect will be observed and measured by calculating the fresh weight of samples after the specified time period has been elapsed.

Light: Algal cultures will be placed out door in open sunlight, inside the lab near window and under artificial light provided by fluorescent tubes. The effect of light on the growth of algae will be calculated by measuring the algal biomass.

Aeration/mixing: Different means of mixing will be used for this purpose. For mixing orbital shaker OS 5 basic yellow line will be used and the flasks will be kept over the shaker at 300 rpm. Aeration will be supplied through aerating pumps. These cultures will be placed indoor where constant sunlight can be given. The growth results will then be compared and cultures with maximum growth will be continued.

Strain Improvement

The screened algal isolates will be improved for their strains for the following properties: 1) Increased Growth Rate, 2) Increased Stress Tolerance, 3) Increased Substrate Utilization, and 4) Increased Product Yield. Natural selection of improved strains will be done by selective adaption methods.The production media will be optimised for better yields and natural selection.

Lipid/oil harvesting

Oil Extraction: Bligh and Dyer (1959) extraction method will be performed to extract the lipids from algae. All the three types of algae (Spirogyra, Oedogonium and Chlorella.) were extracted by using this method.

Characterisation of lipis/oils by modern analytical technique

Modern analytical methods like GC-MS, HPLC, NMR will be employed for the characterisation of the lipids/oils extracted from algae.

Chromatography is one of the most powerful analytical procedures for separating and analyzing the properties of lipids, especially when combined with techniques which can be used to identify the chemical structure of the peaks.

Significance of Algal Biofuel / Algal Oil

India is now going through an unprecedented energy crisis and no one seems to be certain as to how the country would come out of the situation. The present import of crude oil in India is around 180 million tonnes per annum. The net result of this scenario would be that Indian industry and economy would become highly vulnerable to international price and supply pressure for crude oil, coal and natural gas in the near future. To avoid this situation, the country has to urgently find an appropriate alternate source of energy in tune with the country's strength, geographical conditions and the need.

It is indeed surprising that India has not seriously considered the algae biofuel as an alternative means of energy. More than 99 per cent of commercial algae biomass produced worldwide currently is mainly from seaweeds farmed near the seashore. India should not let go the opportunity to utilise the algae, that can be cultivated in large quantity in the Indian coastal regions. Algae can be used as a source for biofuel and bioethanol; apart from this, algae can also be used for the production.

Biofuels are a renewable and sustainable alternative energy source; different regions of the world have used them to partially replace the use of fossil fuels, such is the case of Brazil with sugarcane, Europe and parts of Asia using mostly palm oil as their production source and for the case of biofuels produced from microalgae, countries like Brazil, Japan, China and the Unites States are the leaders in the field. Biofuel production is an important source of job creation, and the first generation of biofuels has allowed significant increments in farmers' incomes.

Advantages for India

Algae is the third-generation biofuel and can be an ideal solution for India's impending fuel crisis, as India's long coastal region and tropical climate can facilitate the cultivation of algae in India in mass scale. This calls for strong and dedicated efforts from Government and industries. The several advantageous salient features of algae in Indian conditions include the following: vast coastline, sufficient solar energy, does not compete with food crops for land availability

International Status

The rapid growing population of the world continuously increases the global demand for fuel energy. The intensive use of fossil fuels worldwide leads to its depletion and will bring them close to the point of exhaustion due to unsustainable and non-renewable nature. Thus, biofuels are now a growing opportunity throughout the world as alternative to fossil fuels. Some developed countries are already producing biofuels at the commercial level. Biofuels such as biodiesel and bioethanol are proving to be excellent alternative fuels and can be produced from several resources of biomass, such as food crops, crop wastes or fruits, woody parts of plants, garbage, and algae. Most of the microalgae species are favourable for biodiesel production due to high lipids contents. Global production of biofuels has increased from 4.8 to 16.0 billion of gallons from 2000 to 2007. Currently, the USA and Brazil are the world leaders in the production of bioethanol. Their contribution is approximately 75–80% of the world total bioethanol production. The USA have 187 commercial bioethanol plants, which mainly produce bioethanol from corn grain. In 2013, Brazil produced 37 billion L of bioethanol using sugar cane as the main feedstock. The European Union (EU) uses wheat and sugar beet as the preferred feedstock for bioethanol production and produces 2.0 billion gallons annual.

National Status

In 2001, India started a modest 5% ethanol blending pilot programme. The biofuel mission was adopted in 2003 with the launch of the National Mission on Biodiesel that sought to achieve 20% biodiesel blending in diesel by 2011–12. This was followed by the National Policy on Biofuel (NPB) in 2009 and subsequently the revised NPB was launched in 2018.

The NPB 2009 envisaged an optional blending target of 20% for both ethanol and biodiesel by 2017. The objective of NPB 2009 was to facilitate the optimal development of domestic biomass feedstock for biofuel production. The NPB 2009 laid down the vision, goals and strategy for the development of biofuels and proposed an enabling framework of financial, institutional and technological interventions.

The revised policy on biofuels, NPB 2018, became effective from May 16, 2018, and proposed an indicative blending target of 20% ethanol in petrol and 5% biodiesel in diesel by 2030.

Proposed work plan (year wise plan of work and targets to be achieved)

First Year :

1. Oil producing algae isolation, identification, and characterisation
2. Screening of oil producing potentials
3. Optimisation of oil production for better yield

Second Year :

1. Strain improvement for natural selection and selective adaptation method
2. Lipid / Oil harvesting

Third Year :

1. Characterisation of Lipid / Oil by modern analytical methods
2. Deposition of culture in Government Depositories and generating the accession no.
3. Pilot scale production of oil by pure culture

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Signature of Guide Signature of Research Student

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